Pleural Macrophage Recruitment and Activation in Asbestos-induced Pleural Injury

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The pathogenesis of asbestos-induced pleural fibrosis is poorly understood. Moreover, there has been a long-standing controversy regarding the relative potential of different commercial types of asbestos to cause pleural disease. We postulated that inhaled asbestos fibers translocate to the pleural space where they stimulate the recruitment and activation of pleural macrophages. To test this hypothesis, and to determine whether there are differences between inhaled amphibole and serpentine asbestos, Fischer 344 rats were exposed by intermittent inhalation (6 hr/day for 5 days/week over 2 weeks) to either National Institute of Environmental Health Sciences (NIEHS) crocidolite (average concentration 7.55 mg/m^3) or NIEHS chrysotile fibers (average concentration 8.51 mg/m^3). Comparisons were made with sham-exposed rats. The rats were sacrificed at 1 and 6 weeks after the cessation of exposure. More pleural macrophages were recovered at 1 and 6 weeks after crocidolite and chrysotile exposure than after sham exposure. Small numbers of crocidolite fibers (approximately 1 per 4000 cells) were detected in the pleural cell pellet of one crocidolite-exposed rat by scanning electron microscopy. Pleural macrophage supernatants were assayed for production of nitric oxide (NO) (by the Griess reaction) and tumor necrosis factor alpha (TNF-α) (by an enzyme-linked immunosorbent assay method). Significantly greater amounts of NO as well as TNF-α were generated by pleural macrophages at 1 and 6 weeks after either crocidolite or chrysotile inhalation than after sham exposure. Conceivably, translocation of asbestos fibers to the pleural space may provide a stimulus for persistent pleural space inflammation, cytokine production, and the generation of toxic oxygen and nitrogen radicals. Enhanced cytokine secretion within the pleural space may in turn upregulate adhesion molecule expression and the synthesis of extracellular matrix constituents by pleural mesothelial cells. Thus, our findings may have significance for the development of asbestos-induced pleural injury. — *Environ Health Perspect* 105(Suppl 5):1257–1260 (1997)

Key words: asbestos, pleural fibrosis, pleural macrophage, tumor necrosis factor alpha, nitric oxide, cytokines

Introduction

Parietal pleural plaques and/or visceral pleural fibrosis are the most common clinical manifestations of asbestos-related disease (*1*). Although all varieties of asbestos are capable of causing pleural fibrosis, there has been considerable debate regarding the relative potential of different commercial types of asbestos to induce pleural injury. Furthermore, despite major advances in our understanding of the mechanisms governing the development of asbestosis, the pathogenesis of asbestos-related pleural fibrosis remains poorly understood (*1–3*). There is some evidence that fibers can translocate to the pleural region after *in vivo* asbestos exposure although the precise routes of translocation remain undefined. Analyses of digested tissues obtained from patients with diffuse pleural fibrosis, pleural plaques, and anthracotic black spots in the parietal pleura reveal the presence of asbestos fibers at these sites (*4–7*). However, those studies afforded no consistent picture as to whether chrysotile or amphibole asbestos represents the predominant fiber type in the parietal pleura of asbestos-exposed individuals.

Although these analytic studies of pleural fiber burden may explain why asbestos exposure causes pleural fibrosis, they provide no insight as to how inhaled asbestos fibers may evoke pleural injury and which cell types may be involved. Conceivably, inhaled fibers may translocate to the pleural space where they are phagocytized by pleural mesothelial cells and/or pleural macrophages. The presence of asbestos fibers in the pleural space may in turn stimulate the recruitment and activation of pleural macrophages in a manner analogous to that shown for alveolar macrophages at sites of asbestos deposition within the respiratory bronchioles (*8,9*). This study was designed to test this hypothesis in a rat asbestos inhalation model.

Materials and Methods

Inhalation Exposure Regimen

Three groups of male Fischer 344 rats were placed in inhalation chambers and exposed to either National Institute of Environmental Health Sciences (NIEHS) crocidolite asbestos fibers (time-weighted average concentration 7.55 mg/m^3), NIEHS chrysotile asbestos fibers (time-weighted average concentration 8.51 mg/m^3), or filtered room air (sham-exposed group). The rats in each group were comparably matched for age (47 days old) and size (mean group weights 149–151 g) prior to inhalation exposure. Fiber aerosols were created using a modified Timbrell generator (BGI, Waltham, MA) (*10*). Each group was exposed for 6 hr/day, 5 days/week for 2 weeks. The airborne fiber-size distributions were determined by a Sierra cascade impactor (Andersen Instruments, Atlanta, GA). The

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Abbreviations used: DMEM, Dulbecco modified Eagle medium; EDXA, energy-dispersive X-ray analysis; HBSS, Hanks balanced salt solution; NIEHS, National Institute of Environmental Health Sciences; NO, nitric oxide; SEM, scanning electron microscopy; TNF-α, tumor necrosis factor alpha.

asbestos aerosols were respirable (mass median aerodynamic diameter for crocidolite 0.45 μm, geometric SD 3.30; mass median aerodynamic diameter for chrysotile 0.57 μm, geometric SD 4.39).

Pleural Lavage Analyses

Rats were sacrificed with 50 mg/kg sodium pentobarbital intraperitoneally, followed by aortic exsanguination, at 1 and 6 weeks after the cessation of exposure. Pleural lavage was performed by inserting an 18-gauge Teflon cannula (Becton Dickinson, Franklin Lakes, NJ) into the pleural cavity via a small diaphragmatic aperture. Each pleural cavity was lavaged twice *in situ* with 5 ml Ca^{++}- and Mg^{++}-free Hanks balanced salt solution (HBSS), which was prewarmed to 37°C (*11*). After two washes in Ca^{++}- and Mg^{++}-free HBSS, the pleural cells were suspended in serum-free Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (2.5 μg/ml). Differential cell counts were performed on cytospin preparations stained with Diff-Quik (Baxter Healthcare, McGaw Park, IL). No significant differences were observed between the three exposed groups with respect to the percentages of pleural cells obtained: these comprised approximately 62 to 76% macrophages, 15 to 20% mast cells, 8 to 13% eosinophils, and < 3% neutrophils.

For mineral fiber analysis, pleural cell pellets from individual rats were fixed in 0.2% glutaraldehyde in 0.2 M phosphate buffer. Both cell pellets and pleural lavage fluid samples were prepared for scanning electron microscopy (SEM) analysis by digestion in hypochlorite, as described previously (*12*). The residue was collected on 0.4 μm (pore size) polycarbonate filters, mounted on a carbon disc with colloidal graphite, then sputter-coated with gold. Mineral fibers were analyzed by SEM and their chemical composition was determined by energy-dispersive X-ray analysis (EDXA).

Pleural Cell Cultures

To determine cytokine and nitric oxide (NO) production by pleural macrophages, the pleural cells obtained from two to three rats were pooled, suspended, and added to 24-well tissue culture plates (2×10^6 cells/well). After the cells were allowed to attach for 1 hr at 37°C, nonadherent cells were removed, whereupon 1 ml fresh DMEM was added and cultures were incubated for an additional 24 hr at 37°C in a humidified environment of 5% CO_2. Thereafter, conditioned medium samples were collected and stored at −80°C. Tumor necrosis factor alpha (TNF-α) in conditioned medium was measured by an enzyme-linked immunosorbent assay using a TNF-α test kit (Genzyme, Cambridge, MA). Determination of NO in conditioned medium was performed by measurement of its oxidation product, nitrite ion, by the Griess reaction, as described previously (*13*).

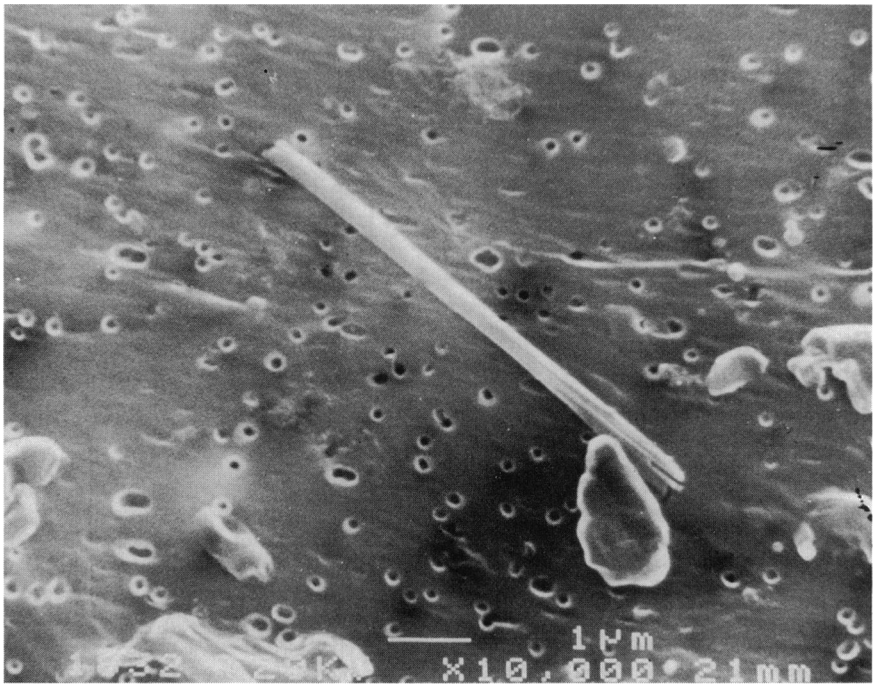

Figure 1. Scanning electron micrograph of a fiber detected in the pleural cell pellet of a crocidolite-exposed rat.

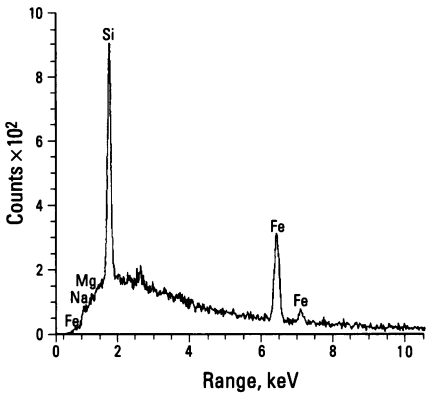

Figure 2. Chemical composition by EDXA of the fiber seen in Figure 1 is consistent with that of crocidolite.

Data Analysis

The results of the pleural cell count analyses and the determinations of NO and TNF-α production were expressed as the mean + SEM of three experiments. Statistical comparisons were performed using a two-tailed Student's *t*-test for unpaired samples.

Results

Fiber analyses were performed only on rats sacrificed at 1 week after the cessation of exposure. No fibers were detected in the pleural lavage fluid obtained from any of the exposed rats. However, several fibers were detected by SEM analysis of the pleural lavage cell pellet from one of three crocidolite-exposed rats examined. The fibers were less than 0.5 μm in diameter and approximately 8 to 10 μm in length (Figure 1). In each instance, the characteristic elemental composition of crocidolite was identified by EDXA (Figure 2). The number of cell-associated crocidolite fibers corresponded to approximately 1 fiber/4000 pleural cells. No fibers were detected in the pleural lavage cell pellets from sham-exposed animals. Fiber analysis was not performed on any chrysotile-exposed rats.

Distinct differences were observed between sham-exposed and asbestos-exposed rats with respect to the total numbers of leukocytes and the numbers of macrophages recovered by pleural lavage at both 1 and 6 weeks after the cessation of exposure (Table 1). Thus, the total number

Table 1. Distribution of pleural lavage cell populations obtained after sham, crocidolite, and chrysotile exposure.

Lavage cell type	1 week after cessation of exposure[a]			6 weeks after cessation of exposure[a]		
	Sham	Crocidolite	Chrysotile	Sham	Crocidolite	Chrysotile
Total number	3.2	5.75	4.83	8.82	13.14	12.6
Macrophages	2.27	4.08	3.67	6.21	9.18	7.84
Neutrophils	0.03	0.09	0.04	0.03	0.06	0.06
Lymphocytes	0.19	0.14	0.11	0.08	0.19	0.17
Eosinophils	0.33	0.57	0.33	0.72	1.1	1.45
Mast cells	0.38	0.87	0.68	1.78	2.61	3.08

[a]Pleural lavage cell yield $\times 10^6$. Mean of pooled data from three rats/group at each time point.

of lavagable leukocytes at 1 week after the cessation of exposure was significantly greater in the crocidolite-exposed group than in the sham-exposed group ($p < 0.01$). A similar trend, albeit statistically insignificant, was noted after chrysotile inhalation. Rats from all three exposure groups yielded an age-related finding: greater numbers of total lavagable leukocytes at 6 than at 1 week after exposure (Table 1). However, 6 weeks after cessation of exposure, the total number of lavagable leukocytes was still significantly greater after either crocidolite ($p < 0.0005$ vs sham exposure) or chrysotile asbestos inhalation ($p < 0.05$ vs sham exposure) than after sham exposure. At 1 and 6 weeks after cessation of exposure, the number of pleural macrophages recovered increased after asbestos exposure, especially in the crocidolite-exposed group (Table 1). Although the number of neutrophils also increased at 1 and 6 weeks after both types of asbestos exposure, these cells represented only a minor fraction (< 3%) of the total lavageable leukocytes.

Cultured pleural macrophages from both groups of asbestos-exposed rats displayed a heightened propensity to secrete cytokine, TNF-α, and generate NO radical. Thus, significantly greater amounts of TNF-α were produced by pleural macrophages at 1 week after the termination of either crocidolite or chrysotile exposure than after sham exposure (Figure 3). This effect persisted for as long as 6 weeks after cessation of exposure. Similarly, formation of NO was upregulated significantly in pleural macrophages obtained from crocidolite-exposed and chrysotile-exposed rats 1 and 6 weeks after exposure ceased (Figure 4).

Discussion

This study showed in a rat model that inhalation of either amphibole or serpentine asbestos fibers induces a macrophage inflammatory response within the pleural space that persists for at least 6 weeks after the cessation of asbestos exposure. These findings corroborate those of an earlier study that demonstrated that intrabronchially instilled amosite fibers stimulated the recruitment of newly arrived macrophages to the pleural space (*14*). Conceivably, translocation of asbestos fibers to the pleural space may provide the initial stimulus for pleural macrophage recruitment. The detection of multiple crocidolite asbestos fibers in the pleural cell pellet from a crocidolite-exposed rat provides strong support for this hypothesis. To our knowledge, this is the first documentation that inhaled asbestos fibers can migrate to the pleural space. It is of interest that the crocidolite fibers detected were in the potentially carcinogenic (i.e., ≥ 8 μm in length) distribution range. This contrasts with the findings of Viallat et al. (*15*), who detected chrysotile fibers with a mean length of 0.44 to 1.32 μm in pleural lavage fluid after intratracheal injection in rats.

This study also showed that inhalation of asbestos fibers upregulated TNF-α production by rat pleural macrophages. These effects were observed after crocidolite as well as chrysotile inhalation. Enhanced production of TNF-α within the pleural space may have relevance to the induction of pleural injury, as this cytokine upregulates murine mesothelial intercellular adhesion molecule-1 adhesion molecule expression and increases the adhesive affinity of murine mesothelial cells for peritoneal mononuclear leukocytes (*16*). Also, TNF-α stimulates rat pleural mesothelial cell proliferation and collagen synthesis (*17*).

We previously showed that both amphibole and serpentine asbestos fibers stimulated the formation of the NO radical by rat alveolar macrophages *in vitro* (*12*). Furthermore, the effects of crocidolite and chrysotile were synergistic with that of interferon-γ. This study extends those findings to the *in vivo* environment, where we demonstrated persistent, upregulated production of NO by pleural macrophages after either crocidolite or chrysotile inhalation. Although there is no obvious explanation for this enhanced generation of pleural macrophage NO, it is conceivable that upregulated secretion of pleural cytokines (e.g., TNF-α) may play a role. Asbestos-induced NO formation may mediate pleural injury via a variety of mechanisms including generation of peroxynitrite anion (which can initiate lipid peroxidation, oxidize sulfhydryl moieties, and damage membrane channels), nitrosation of iron-containing cellular enzymes, and induction of DNA damage and mutations (*18–21*).

In summary, this study demonstrated that inhaled asbestos fibers can translocate to the pleural space and that crocidolite as well as chrysotile asbestos inhalation can stimulate the recruitment and activation of pleural macrophages. Our findings also suggest that inhaled serpentine (chrysotile) and amphibole (crocidolite) asbestos fibers may have a similar capacity to induce pleural space inflammation. Because of the close anatomic proximity between pleural macrophages and pleural mesothelial cells, it is conceivable that persistent, upregulated pleural cytokine and NO radical production may be implicated in the pathogenesis of asbestos-related pleural injury. Although this study has focused exclusively on the possible roles of TNF-α and NO in asbestos-induced pleural injury, it is likely that a variety of other cytokines and growth

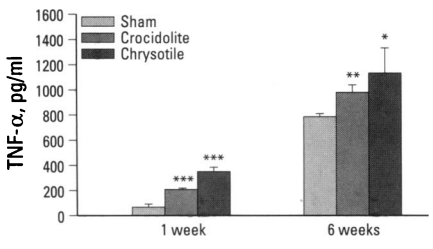

Figure 3. TNF-α production by pleural macrophages from sham-exposed, crocidolite-exposed, and chrysotile-exposed rats at 1 and 6 weeks after the cessation of inhalation exposure. *$p < 0.05$ vs sham; **$p < 0.005$ vs sham; ***$p < 0.0001$ vs sham.

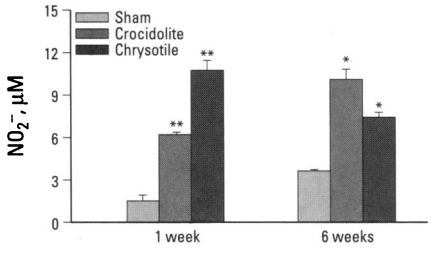

Figure 4. Nitric oxide (NO_2^-) formation by pleural macrophages from sham-exposed, crocidolite-exposed, and chrysotile-exposed rats at 1 and 6 weeks after the cessation of inhalation exposure. *$p < 0.005$ vs sham; **$p < 0.0005$ vs sham.

factors may be implicated in this process (*3*). Notably, pleural mesothelial cells are capable of secreting the cytokines interleukin-8 and monocyte chemoattractant protein-1, which are considered important in pleural space inflammation and which may play a role in the recruitment of leukocytes to the pleural space (*22–24*). Additionally, asbestos fibers upregulate the production of the fibroblast chemotaxin and growth factor, fibronectin, by pleural mesothelial cells (*25*). This finding may have significance for the reparative response of the pleura to asbestos-induced injury.

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